*Journal of Chromatography*, 390 (1987) 458–462
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM 19 253

## Note

# High-performance liquid chromatographic assay for hexokinase

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(First received September 9th, 1986; revised manuscript received November 6th, 1986)

Hexokinase (HK) is a phosphorylating enzyme involved in respiration and fermentation processes[1]. Its main application is the determination of glucose, fructose and mannose using ATP as a phosphate donor. The activity of hexokinase is determined by measuring the change in absorbance at 340 nm per unit time, according to the following reactions:

$$\text{D-glucose} + \text{ATP} \overset{\text{HK}}{\rightleftarrows} \text{D-glucose-6-P} + \text{ADP} \quad (1)$$

$$\text{D-glucose-6-P} + \text{NADP}^+ \overset{\text{G6P-DH}}{\rightleftarrows} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+ \quad (2)$$

However, this spectrophotometric method[2] requires the use of another enzyme, glucose-6-phosphate dehydrogenase (G6P-DH) and the cofactor $NADP^+$.

We succedeed in developing a high-performance liquid chromatographic (HPLC) assay based on the key step (1), *i.e.*, the phosphorylation of glucose. ATP and ADP were separated isocratically on a $C_{18}$ column using 0.05 *M* sodium monohydrogen phosphate (pH 6.1) as eluent, and the amount of ADP produced per unit time and per milligram of protein gave a measure of the hexokinase activity.

## EXPERIMENTAL

### *Materials*

Hexokinase, glucose-6-phosphate dehydrogenase, ATP, ADP, NADP and thymine were obtained from Boehringer (Mannheim, F.R.G.) and triethanolamine and glucose from Carlo Erba (Milan, Italy). Acetonitrile was of HPLC grade (Chromasolv) (Riedel-de Haën, Hannover, F.R.G.). Water was distilled and filtered through a 0.45 μm membrane (Type HA, Millipore). All others chemicals were of analytical-reagent grade.

### *Chromatographic conditions*

The HPLC system consisted of a Model A 590 pump equipped with a Model U6K Universal injector, a Model 440 UV detector and a Model 730 Data Module (Waters Assoc., Milford, MA, U.S.A.). Samples were chromatographated on a μBon-

0021-9673/87/$03.50 

dapak $C_{18}$ column (Waters Assoc.) using 0.05 *M* sodium monohydrogen phosphate (pH 6.1) as eluent. The flow-rate was 2 ml/min and ultraviolet absorption was measured at 254 nm (0.05 a.u.f.s.).

*Calibration graphs*

Into individual test-tubes (1.5 ml) were pipetted known volumes (5–60 μl) of ATP (2.4 m*M*) and ADP (2.0 m*M*) aqueous solutions. Then 400 μl of the internal standard solution (0.2 m*M* thymine in water) were added to each tube and the volume was brought to 1 ml with the eluent. Replicate injection of 10 μl were made for each point ($n = 7$).

*Stability of ATP and ADP solutions*

ATP and ADP solutions (0.2 m*M*) in 0.1 *M* phosphate buffer (pH 2.7) were kept at 25°C for 4 h. At 20 min intervals 10 μl aliquots were injected.

*Assay of hexokinase activity*

To 0.55 *M* glucose in 0.1 *M* triethanolamine buffer (pH 7.55) (1.2 ml) were added 0.1 *M* ATP (0.1 ml) and 0.1 *M* $MgCl_2$ (0.2 ml) solutions. The resulting mixture was diluted with 1.5 ml of triethanolamine buffer, equilibrated at 25°C and then the enzymatic reaction was started by adding 20 μl of hexokinase solution (10 mg/ml, 5–50 μl diluted to 2 ml with cold triethanolamine buffer immediately before the assay). After 4 min, 10 μl of the incubation mixture were diluted with 300 μl of 0.08 *M* thymine in 0.1 *M* phosphate buffer (pH 2.7) and replicate injections of 10 μl were made.

## RESULTS AND DISCUSSION

Although reversed-phase separations of ATP and ADP by gradient[3,4] or ion-pair isocratic elution[5,6] are known, the routine assay of the hexokinase activity by these methods was unsuitable. The use of a gradient required an increased analysis time for equilibration, and mobile phases containing ion pairs reduced the column lifetime. Isocratic elution with acidic buffers[7] was then investigated, and the best resolution of ATP, ADP and thymine (internal standard) was achieved on a μBondapak $C_{18}$ column using 0.05 *M* sodium monohydrogen phosphate buffer (pH 6.1) as eluent (Fig. 1). The pH (in the range of 6.0–6.6) was very important for peak resolution, whereas the presence of an organic modifier such as acetonitrile (1–5%) had an insignificant effect.

The pH of the incubation mixture was lowered by dilution with phosphate buffer (pH 2.7) containing the internal standard to stop the enzymatic reaction. At this pH both ATP and ADP were found to be stable for up to 4 h.

Linear relationships between the peak-area ratios (ATP/I.S. and ADP/I.S.) and the amount of ATP and ADP injected were found for the ranges 1.35–0.24 nmol and 1.35–0.05 nmol, respectively:

$$y = 3.89\,x - 0.178 \qquad (r = 0.996)$$

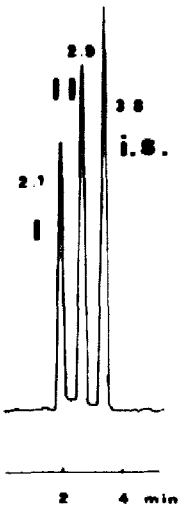


Fig. 1. Typical chromatogram of ATP (I), ADP (II) and thymine (I.S.). Chromatographic conditions: column, μBondapak $C_{18}$ (30 cm × 4 mm I.D.); Eluent, 0.05 *M* sodium monohydrogen phosphate buffer (pH 6.1); flow-rate, 2 ml/min; detection, UV (254 nm).

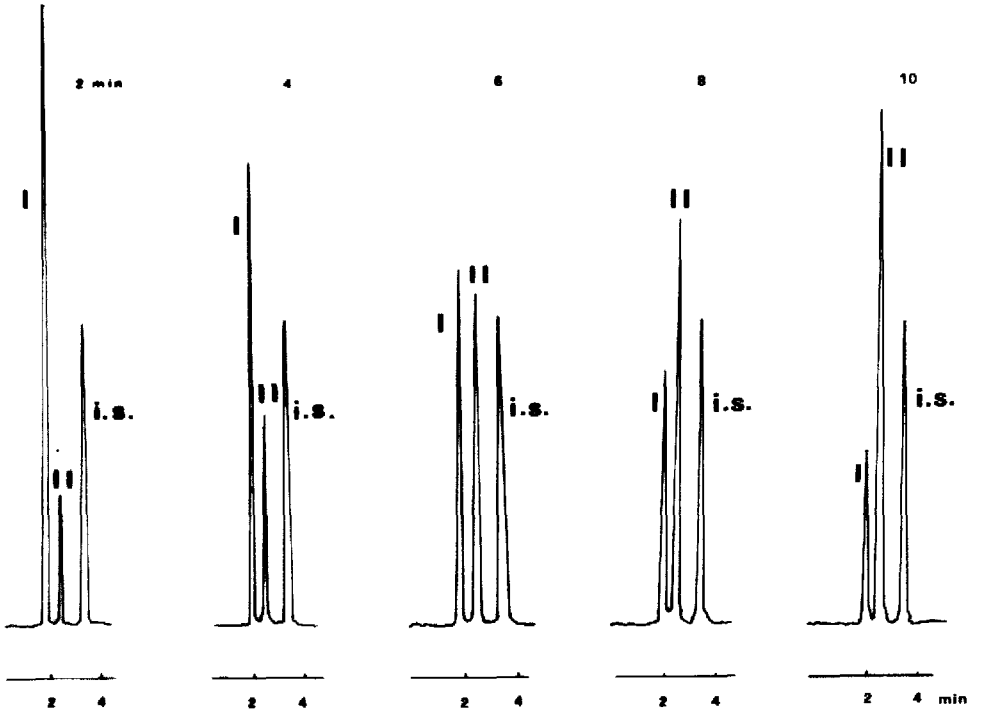


Fig. 2. Chromatograms of the reaction mixture after increasing incubation times. Chromatographic conditions as in Fig. 1.

where $y$ = ATP/I.S. and $x$ = nmoles of ATP injected, and

$$y = 3.26\ x - 0.0088 \qquad (r = 0.999)$$

where $y$ = ADP/I.S. and $x$ = nmoles of ADP injected.

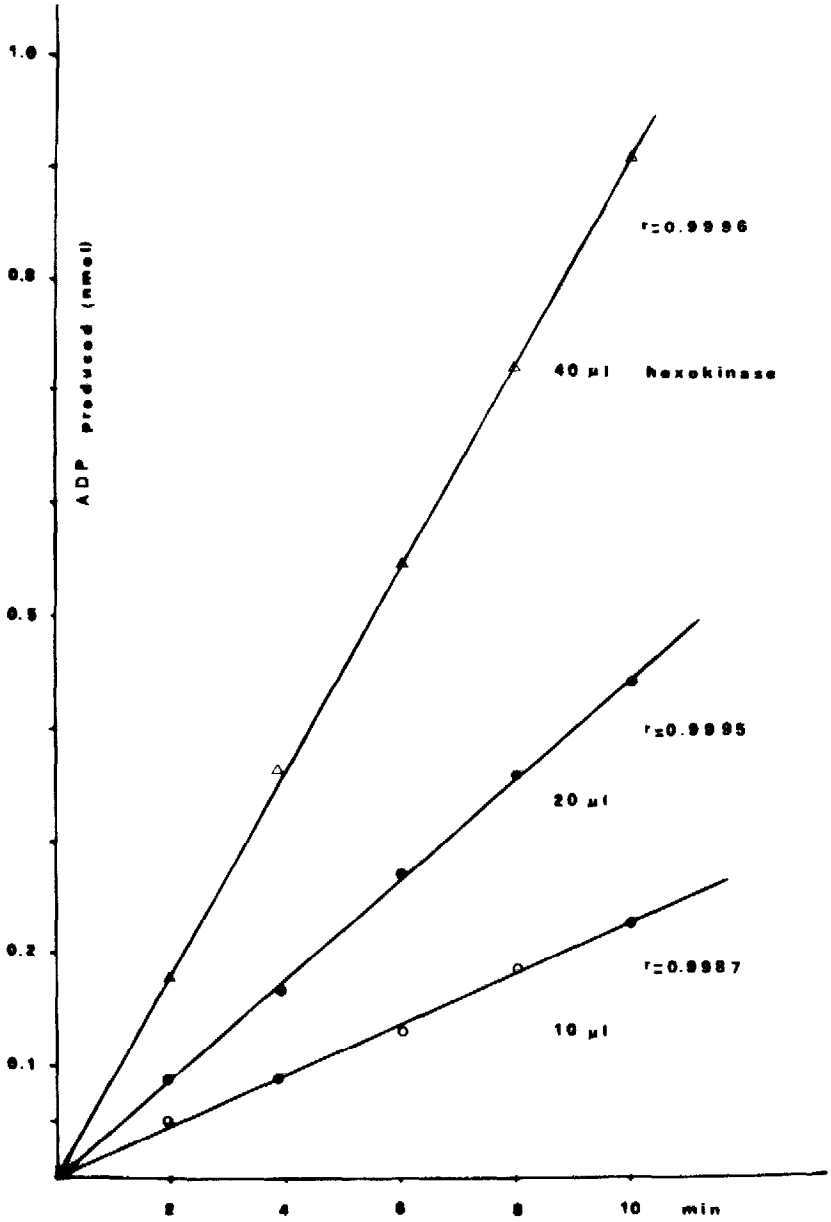


Fig. 3. Correlation of the amount of ADP produced with the volume of hexokinase employed. (10 $\mu$l corresponding to 0.5 $\mu$g of hexokinase).

The enzymatic reaction caused a significant disappearance of ATP and production of ADP (Fig. 2); in a blank incubation ATP was not converted into ADP.

As shown in Fig. 3, the amount of ADP produced was linearly dependent on the hexokinase activity in the range 0.15–1.5 μg/ml hexokinase and on the incubation time up to 10 min. Therefore, the assay was routinely performed stopping the reaction after 4 min. The activity of hexokinase was calculated from the enzymatically produced ADP using the equation

$$\text{specific activity } (\mu\text{mol/min} \cdot \text{mg}) = \frac{\mu\text{mol/min of ADP produced}}{\text{mg of hexokinase}}$$

Different commercial samples of hexokinase were assayed and reproducible values were obtained with a standard deviation of 2.1%. The results correlated well with those obtained by the spectrophotometric method, the correlation coefficient being 0.98.

In conclusion, the potential of HPLC for a rapid and economic assay of hexokinase has been demonstrated. The extension of this procedure to the determination of glucose and ATP in complex matrices is under investigation.

ACKNOWLEDGEMENT

The authors acknowledge the technical assistance of S. De Alberti.

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